IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: HARPOLD et al. Group Art Unit: 1812

Serial No.: 07/938,154 Examiner: Ulm, J.

Filed: April 3, 1991

For: HUMAN NEURONAL NICOTINIC ACETYL CHOLINE RECEPTOR COMPOSITIONS AND METHODS EMPLOYING SAME

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, STEVEN BRADLEY ELLIS declare as follows:

1) I am a co-inventor of the above-identified U.S. Patent Application Serial No. 07/938,154 as well as the parent application U.S. application Serial No. 07/504,455, which is now U.S. Patent No. 5,369,028.

2) I was employed by THE SALK INSTITUTE BIO-TECHNOLOGY/INDUSTRIAL ASSOCIATES, INC. (hereinafter SIBIA) from December 1981 through July, 1995. I served as a Molecular Cellular Biologist from 1981 through 1985, as a Research Scientist from 1985 through April 1992, and as a Senior Research Scientist from 1992 to 1995.

3) As the details presented below indicate, it was not a straight-forward endeavor to obtain DNA encoding any of the human neuronal nicotinic acetylcholine receptors (NAChRs) despite the fact that DNA encoding certain rat nicotinic acetylcholine receptor subunits were known. In attempting to clone the subunits, a number of difficulties were encountered, including: a) the difficulty in obtaining suitable human neuronal tissue samples from which to prepare libraries; b) the differences between the distribution of receptor subtypes in humans and rats; and c) the unpredictability of such tissue samples with respect to the types of receptors that are expressed.

4) The following description of cloning of each of the receptor subunits evidences the above-described difficulties and the differences between the rat NAChR subunits and human NAChR subunits.

ISOLATION OF DNA ENCODING A HUMAN NAChR α_2 SUBUNIT

RNA encoding the rat α_4 subunit is abundant in rat thalamus. Consequently, in order to isolate DNA encoding a human NAChR α_4 subunit, a human thalamus library was screened with DNA encoding the rat α_4 subunit as a probe. Four positive DNA clones $\alpha_{2.1}$, $\alpha_{2.2}$, $\alpha_{2.11}$, and $\alpha_{2.13}$ were obtained. The four clones, however, did not have substantial homology with rat α_4-encoding but were more homologous to rat α_2-encoding DNA. Clones $\alpha_{2.1}$ and $\alpha_{2.13}$ were spliced to produce a full length α_2-encoding clone. The 3' end of human clone $\alpha_{2.1}$ is about 81% homologous to rat α_2-encoding DNA and only 43% homologous to the DNA clones encoding the rat α_3, α_4 and β_2 subunits. The human clone $\alpha_{2.11}$ is only about 75% homologous to the DNA encoding the rat α_2 subunit. Thus, the resulting clone is less than about 80% homologous to the DNA encoding rat α_2.

ISOLATION OF DNA ENCODING A HUMAN NAChR α_3 SUBUNIT

To isolate the NAChR α_3 subunit the following five human cDNA libraries were screened using the rat α_3 clone as a probe:

Library screened with rat α_3 probe	Positives
SIBIA prefrontal cortex library (9.4×10^5 recombinants)	0
Clontech Temporal Cortex library (9.2×10^5 recombinants)	0
ATCC Basal Ganglia library (5×10^5 recombinants)	0
ATCC Spinal Cord library (5×10^5 recombinants)	0
ATCC Brain Stem library (6.6×10^5 recombinants)	7

Thus, the only clones obtained, clones, $\alpha_{3.1}$ -$\alpha_{3.7}$, were obtained from the brain stem library. In rats, however, α_3 is not abundant in the brain stem. The rat α_3 encoding DNA had been isolated from a rat PC12 cell-derived cDNA library and the rat α_3 subunit appears to be a CNS-associated subunit that is abundantly expressed in the thalamus. Since RNA encoding the α_3 subunit is abundant in rat thalamus, a human thalamus library was screened with $\alpha_{3.6}$. Only four positive clones $\alpha_{3.11}$- $\alpha_{3.14}$ were obtained.

The full-length human nicotinic acetylcholine receptor α_3 subunit was ultimately isolated by screening a cDNA library prepared from mRNA isolated from dibutyryl cyclic AMP-induced IMR32 (human peripheral nervous system neuroblastoma) cells, using rat-human hybrid α_3 probe (550 nucleotides containing the DNA encoding the rat signal sequence and the first five codons of the mature protein and the remainder was from the human α_3 clone $\alpha_{3.5}$). Although IMR32 cells reportedly express few, if any, functional nicotinic acetylcholine receptors (see, e.g., Gotti et al. ((1986) Biochem. Biophys. Res. Commun. 137: 1141-1147, and Clementi et al. (1986) J. Neurochem. 47: 291-297), twenty-four positive clones were obtained. Among the twenty-four clones, two clones had no homology to any known NAChR subunit-encoding DNA and one clone $\alpha_{3.24}$ encodes a full-length α_3 subunit.

The human $\alpha_{3.5}$ clone was used to probe northern blots of RNA from dibutyryl cAMP-induced IMR32 cells and from uninduced IMR32 cells. A 3.2 kb transcript, which is of sufficient size to encode the human α_3 mRNA, was detected in both RNA samples. An additional 4.2 kb transcript was detected in the RNA from the induced cells but not from the uninduced cells.

Human α_3-encoding DNA and a pool of 5′ oligomers were subsequently used to screen another library prepared from dibutyryl cAMP induced IMR32 cells. Sequence analysis of the positive clones revealed that three of them were partial clones identical to corresponding portions of the α_3 clone, but that a fourth positive clone KE$\alpha_{3.6}$ was different. The 5′ end of the clone KE$\alpha_{3.6}$ has only 40% sequence identity with the other α_3 clones and the 3′ end has 100% sequence identity with the other clones. In addition, the 5′ end of KE$\alpha_{3.6}$ does not contain a splice acceptor site, suggesting that it does not represent an incompletely spliced transcript. It appears that KE$\alpha_{3.6}$ is the product of alternative splicing of the α_3 gene.

ISOLATION OF DNA ENCODING A HUMAN NAChR β_2 SUBUNIT

Although β_2 is not abundant in rat prefrontal cortex, screening a prefrontal human brain cortex library (9.4×10^5 recombinants; prepared in house at SIBIA) using rat β_2 cDNA yielded a positive clone, $\beta_{2.1}$, which contains about 80% of the coding portion of the human gene. $\beta_{2.1}$ also was used to screen a temporal cortex library (9.2×10^5 recombinants; Clontech). No positives were found in this cortex library, despite the success in obtaining $\beta_{2.1}$ from the first cortex library using the rat clone. Since mRNA encoding β_2 is abundant in rat thalamus, a human thalamus library (1×10^6 recombinants, prepared at SIBIA) was screened with the $\beta_{2.1}$ clone. Only one positive clone, $\beta_{2.2}$ was obtained. A human thalamus library (1.8×10^6) was then constructed using oligo dT and human β_2 specific primers to prepare the cDNA and screened using the 5' end of the rat β_2 clone as a probe. Three clones $\beta_{2.5}$ -$\beta_{2.7}$ that hybridized to the probe were identified. A full-length β_2 clone was constructed by splicing partial clones $\beta_{2.1}$, $\beta_{2.2}$ and $\beta_{2.7}$.

An IMR32 library (1×10^6 recombinants) was screened with the human β_2 probe. As discussed above, human α_3 appears to be abundantly expressed in IMR32 cells lines, and publications discussing the rat receptors indicate that a functional channel requires an α and β. Since α was expressed in IMR32 cells, it was expected that β would also be expressed. Only four hybridizing clones were identified, however, and have not been completely characterized. Northern analysis of IMR32 RNA, using rat β_2 DNA as probe, did not detect any hybridizing mRNA, thus rendering the precise nature of the β clones unclear. It appears that β_2 may be expressed at very low levels in IMR32 cells.

ATTEMPTS TO ISOLATE DNA ENCODING A HUMAN NAChR α_4 SUBUNIT

After failing to isolate human α_4 clones from a thalamus library, a human brain stem library was screened using low stringency wash conditions with the rat α_4 DNA probe. The eighteen most intensely hybridizing positive clones were analyzed. None of eighteen clones encoded an α_4 subunit.

Since in vitro hybridization studies of RNA isolated from rat brain tissue demonstrate that the α_4 transcript is highly expressed in the habenula (see, e.g., Goldman et al. (1987) Cell 48: 965), a human habenula library was screened under high stringency wash conditions using the rat α_4-encoding DNA. The only hybridizing DNA obtained was a human α_2 clone.

ATTEMPTS TO ISOLATE DNA ENCODING A HUMAN NAChR α_5 SUBUNIT

A randomly primed library prepared from RNA isolated from dibutyryl cAMP-induced IMR32 cells was screened under conditions of high stringency with a 1.1 kb fragment including the 5' end of the coding portion of rat α_5-encoding DNA. No hybridizing clones were obtained. High stringency conditions were used in order to avoid isolating α_3 clones.

It has been reported (Chini et al. (1992) Proc. Nat'l. Acad. Sci. 89: 1572, which is attached hereto) that human α_5-encoding clones have been obtained from a library produced from RNA isolated induced IMR32 cells using a roughly 500 bp rat α_5 probe that encompasses the 3' coding region of the rat clone and screening under low stringency conditions.

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I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

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STEVEN BRADLEY ELLIS

Date: _____
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